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THE PATH OF OXYGEN IN PHOTOSYNTHESIS

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March 31, 1950

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THE PATH OF OXYGEN IN PHOTOSYNTHESIS

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ABSTRACT

March 31, 1950

An experiment is described in which an attempt is made to follow the path of oxygen in photosynthesis by the use of O^{18} as a tracer.

For publication in Plant Physiology

(*) While on leave from Department of Chemistry, Washington University, St. Louis, Missouri

(**) The work described in this paper was sponsored by the Atomic Energy Commission.

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A number of years ago, Ruben and co-workers (1) established by a series of interesting tracer experiments the source of the oxygen evolved in photosynthesis. Utilizing the oxygen isotope of mass 18, these workers found that photosynthesis is represented by equation 1 if the oxygen 18 is initially incorporated in the water and by equation 2 if it is initially incorporated in the carbon dioxide:

$$2H_2O^{18} + CO_2^{16} \longrightarrow (H_2CO^{16}) + H_2O^{16} + O_2^{18} \qquad 1.$$

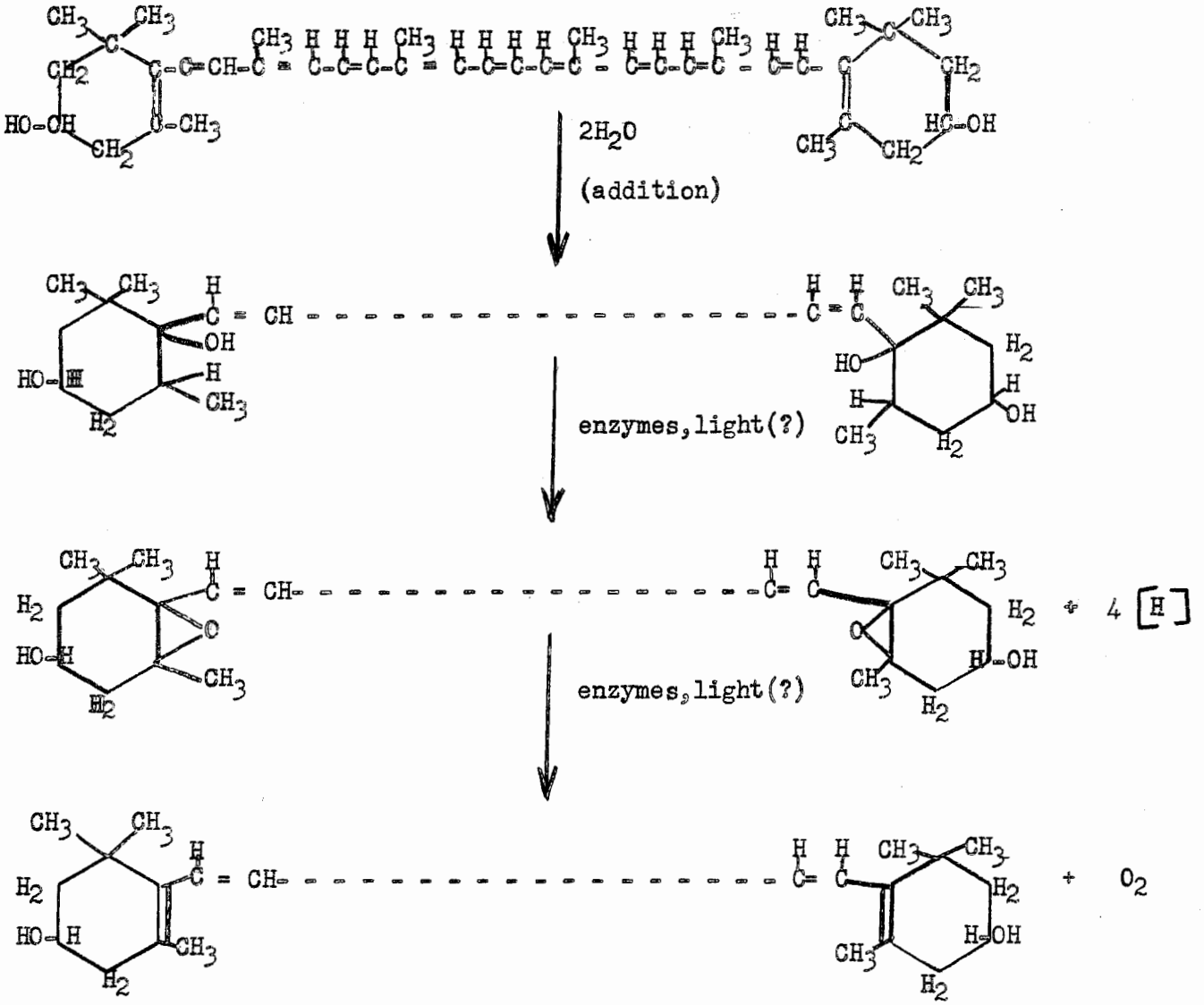
$$2H_2O^{16} + CO_2^{18} \longrightarrow (H_2CO^{18}) + H_2O^{18} + O_2^{16} \qquad 2.$$

It was thus established that the evolved oxygen is derived in its entirety by the oxidation of water.

(1) Ruben, Randall, Kamen and Hyde, J. Am. Chem. Soc., 63, 877 (1941).

In the intervening years, no information has been obtained concerning the manner in which this oxidation takes place, for no oxygen containing compound has ever been established as an intermediate between water oxygen and molecular oxygen. The isolation by Karrer (2) of a number of epoxide structures among the oxygen containing carotenoids has led to a suggestion that perhaps these pigments are such intermediates. The type of mechanism in which they might function is as follows:

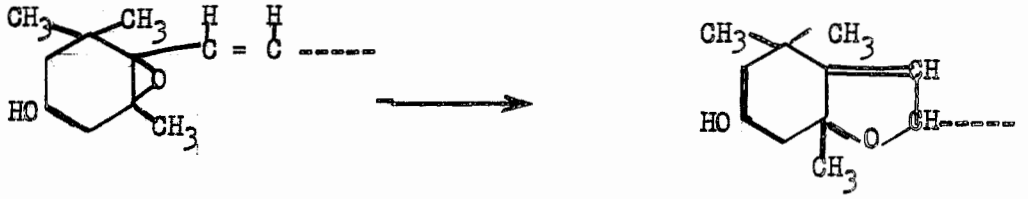
(2) Karrer and Jucker, "Carotinoide" Verlag Birkhäuser, Basel, 1948.



The overall equation is the oxidation of water with the formation of the reducing power required for the reduction of the CO_2:

$$2H_2O + \text{light, enzymes} \longrightarrow O_2 + 4\,[H]$$

The epoxide structures are readily converted to furan compounds,



which are also found in plants, and these too might be intermediates in the oxidation of water.

A straightforward way of testing such a hypothesis would be to photosynthesize a plant of high carotenoid content with water containing oxygen 18, isolate and purify the various epoxide and furan carotenoid pigments, and determine whether or not these pigments contain a higher percentage of oxygen 18 than an identical sample kept in the dark. Such an experiment has been attempted on green algae; it is described in detail in the next section.

EXPERIMENTAL

Analysis of H_2O^{18}

A necessary prerequisite for the experiment is an analysis of the oxygen 18 water to be used in the photosynthesis. The analysis is performed by equilibrating a small sample of CO_2 with a large excess of water, and measuring the O^{18}/O^{16} ratio of the equilibrated CO_2 on a mass spectrograph. (Various difficulties prevent the direct determination of water in the mass spectrograph.) The mass ratio measured for the equilibrated CO_2 is essentially the same as that for the water. The method employed for the equilibration was similar to that of Cohn and Urey (3).

(2) Cohn and Urey, J. Am. Chem. Soc., 60, 679 (1938).

Photosynthesis of the Algae

Twenty cc. of fresh packed green algae (Chlorella pyrenoidosa) were slurried with 50 cc. of 4% oxygen 18 water. The resulting 70 cc. was divided equally between two large Warburg flasks (150 cc. capacity), each flask containing 0.5 gms. of potassium bicarbonate. One of the flasks was wrapped with a metal covered masking tape so as to make it light tight. The two flasks were placed in the Warburg side by side. Photosynthesis was carried out with both top and bottom illumination (a total of 6 G.E. 300 W spot reflectors) until 46.6 cc. of gas had been evolved from the flask exposed to the light. During the same period, the covered flask evolved 14.8 cc. of gas due to CO_2 evolution from the concentrated bicarbonate solution. The total time of light exposure was five hours. Assuming 10 mg. of xanthophylls (4) in 10 gm. of algae and 46.6 - 14.8 = 32 cc. of oxygen evolved, the ratio of xanthophylls to water consumed in the experiment was:

$$\frac{2.6 \times 10^{-3} \text{ moles of water consumed}}{1.6 \times 10^{-3} \text{ moles of xanthophylls present}} \simeq 150$$

Extraction of the Xanthophyll Pigments

The algae suspensions from the light and dark flasks were treated identically as follows:

The algae suspension was centrifuged, and the water decanted off for analysis as above (distillation required first). The algae were then slurried with 400 cc. of absolute methanol, and two or three gms. of $CaCO_3$ added. After 18 hours the methanol was filtered off, and the algae re-extracted with 200 cc. of benzene for eight hours, and 200 cc. of methanol for 24 hours. The algae residues were practically colorless after this treatment. The total methanol and benzene extracts were evaporated to

(4) The approximate amount found in several trials.

150 cc., and treated with 250 cc. of 12% potassium hydroxide in methanol. After 12 hours this solution was diluted with roughly an equal volume of water, and extracted with peroxide-free ether until the ether extracts were colorless. The ether contained mainly xanthophylls, carotenes, steroids and phytol. The total ether extract was washed five times with water, dried overnight with anhydrous sodium sulfate, and evaporated to dryness at 30-40° C under vacuum (nitrogen used on the capillary). The flask containing the pigments was then pumped overnight under high vacuum (10^{-5} mm.). The carotenoid content of this solid residue was about 7%.

Chromatography of Xanthophylls

The contents of the flask from the previous operation were dissolved in 10 cc. of benzene. Twenty cc. of petroleum ether (b.p. 35-60°) were added, and the solution placed on a 2" x 10" column of tightly packed $CaCO_3$ (Baker and Adams reagent grade) which had previously been wet with a small amount of the same solvent. The vacuum applied to the column for development was about 3" of mercury. After absorption of the material was complete, the column was washed with copious quantities of petroleum ether without benzene. The column appeared as shown in Figure 1.

Elution of the pigments was with methanol. The resulting solution was diluted with an equal volume of dilute aqueous sodium chloride, and extracted with peroxide-free ether. The ether was washed with water, and then evaporated to dryness. This procedure removed methanol soluble impurities in the $CaCO_3$. Direct ether elution of the pigments is not very complete.

The cut shown between the dotted lines in Figure 2 is free of the carotenoids and phytol, but still contains about 60-70% of presumably steroid material. The total xanthophyll content is of the order of 10-12 mg. about half of which is lutein. The other half is composed of four xanthophylls, among which are the furan and epoxide structures.

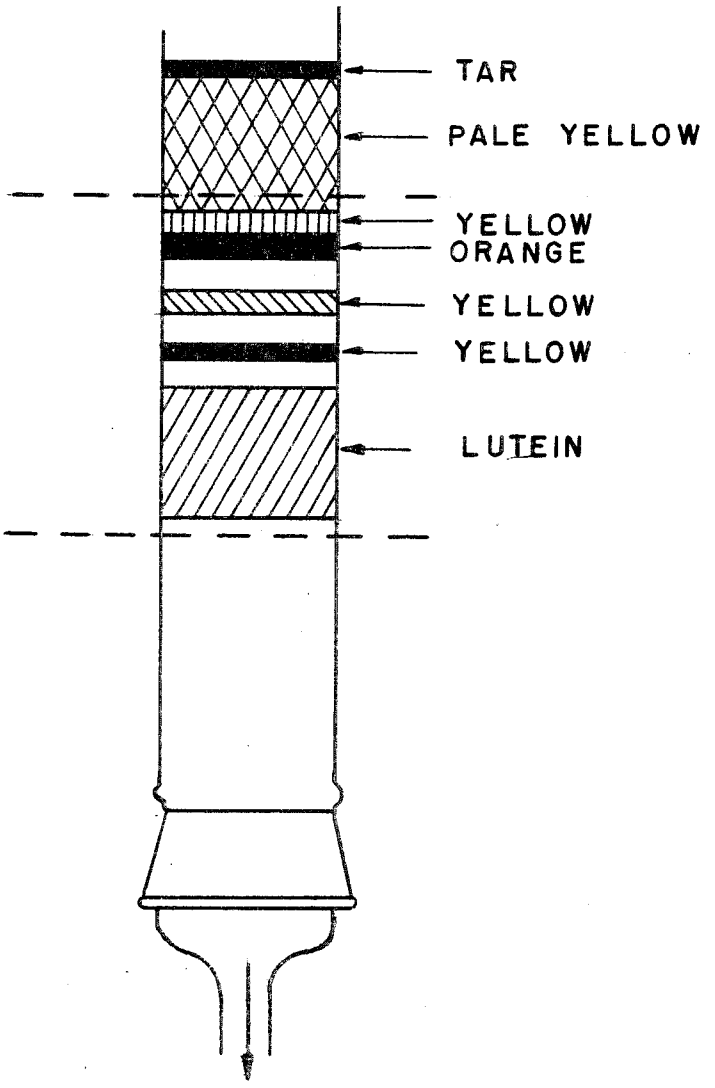

TAR
PALE YELLOW
YELLOW
ORANGE
YELLOW
YELLOW
LUTEIN
CAROTENES

FIG. 1

Mu 116

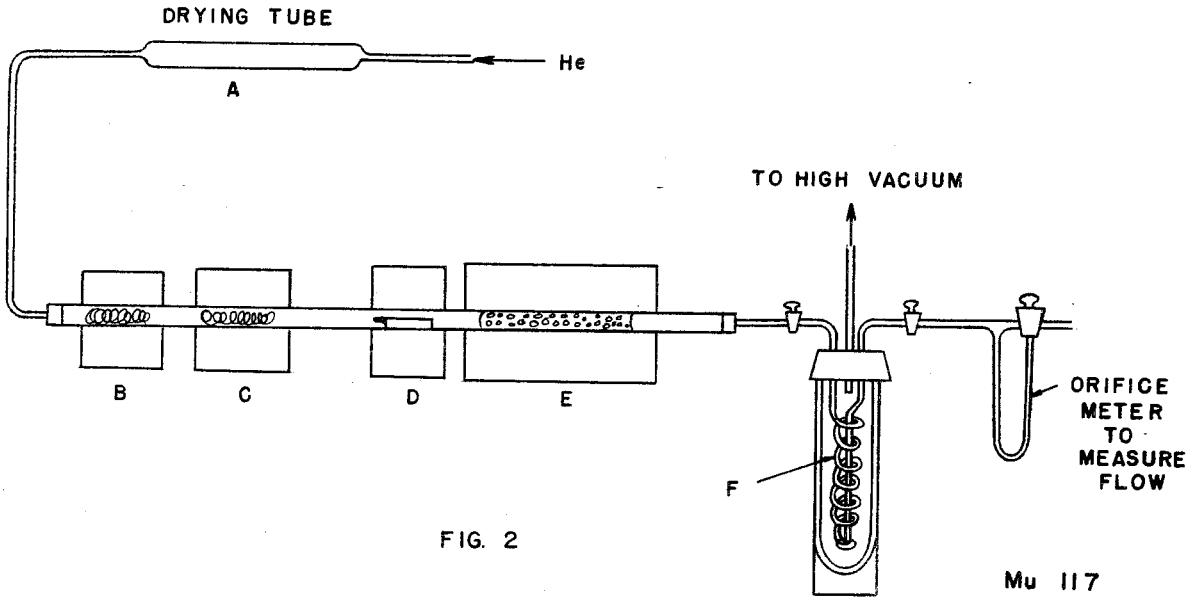

DRYING TUBE
He
A
TO HIGH VACUUM
B
C
D
E
F
ORIFICE METER TO MEASURE FLOW
Mu 117

FIG. 2

Rechromatography of this cut raises the purity to about 50-55%. For separation of the various xanthophylls, a developing solvent of petroleum ether and benzene in the ratio of 2:1 works very well.

Pyrolysis of the Xanthophylls

The oxygen in the xanthophylls must be put in a form suitable for analysis on the mass spectrograph. This was accomplished on a modified combustion train (see Figure 2) as follows: A stream of helium (200 cc./mm.) is passed through a drying tube (A) to remove water, and over two hot (350° C) copper spirals (B) and (C) to remove oxygen.

The sample of carotenoid in a small boat (D) is pyrolysed at 600° C in this stream of dry, oxygen-free helium. The oxygen fragments are all converted to CO by passage over hot carbon (E) at 800° C. The CO is then trapped in the spiral (F) which is maintained at about -240° C by pumping on a dewar flask containing liquid nitrogen with a high speed vacuum pump (Cenco Magavac). (The nitrogen solidifies).

The drying agent used was anhydrous magnesium perchlorate (Dehydrite). The copper spirals were used as insurance, the lack of tarnish in the second spiral showing that the helium was oxygen-free. The carbon used was Fisher CP sugar charcoal, which had to be pumped on at 800° C for 12 hours to complete the decomposition of the sugar. The spiral was packed with coarsely powdered glass to increase the surface area.

It is obvious that before the actual pyrolysis is started, the train must be completely swept out with helium, the various furnaces must be at their correct temperatures, and the spiral trap must be at temperature equilibrium (-240° C).

Separation of Carbon Monoxide from Helium

It was originally planned to analyze the CO directly in the helium mixture, but since the ion current in the spectrograph is limited, most of the current was carried by the helium and the CO peaks were very small. The CO was separated from the helium by absorption of the CO on gas mask charcoal at liquid nitrogen temperatures, and pumping off the helium. The gas mask charcoal was prepared by heating to 120° C and pumping at 10^{-6} mm. for four days.

Spectral Analysis of the Carotenoids

A simple approximate analysis of any carotenoid fraction can be carried out on a Beckman or similar spectrophotometer due to the fact that the carotenoid spectra are all similar in shape and extinction coefficient. The shape is a characteristic three banded system with the highest peak (the middle one) occurring in the region of 4400-4500 Å in methanol solution. The specific extinction coefficient, a, has a value for most carotenoids around 210-240. a is defined:

$$a = \frac{\log_{10} \frac{I_o}{I}}{cl}$$

Where: $\frac{I_o}{I}$ is the reciprocal of the fraction of light transmitted.

c is the concentration, gms./liter

l is the cell thickness in centimeters

DISCUSSION

A considerable number of difficulties were encountered in the experiment which place certain restrictions on the interpretation of the data. The only sample of oxygen 18 water available was one of 50 cc. containing 4% oxygen 18. This amount of water limited the amount of algae which could be run (about 20 cc. of packed cells for division between the light and dark experiments), and this in turn limited the amount of xanthophylls which could be obtained. Now, in 10 cc. of packed cells, there are some 4 or 5 mg. of furan and epoxide carotenoids. This entire amount can be isolated, but it is mixed with roughly an equivalent amount of steroid. To completely free the carotenoid of steroid would mean sacrificing probably three-fourths of the total in chromatography and crystallization. This would leave only about one mg. of carotenoid which would yield one-sixth of the amount of oxygen (as CO) required by the mass spectrograph for analysis. If the oxygen 18 content of the water were high, this one mg. of carotenoid could be diluted with a sufficient amount of a normal oxygen source to give the required amount of CO. However, the oxygen 18 content of the water was rather low, and no advantage could be gained by the difficult purification of the furan and epoxide fractions. This is illustrated by Table I. (Assuming the epoxide and furan oxygens are converted to the same O^{18} content as the water in equilibrium with the algae.)

Table I

A. Estimated dilution factor of the original water if the total carotenoid fraction is used for analysis

Dilution	Estimated factor
Water in algae	0.7
Lutein and other xanthophyll hydroxyl groups	0.3
Steroid material	0.5
Overall dilution	$\simeq$ 0.1

B. Estimation dilution factor of the original water if furan and epoxide xanthophylls are separated and then analyzed.

Dilution	Estimated factor
Water in algae	0.7
Hydroxyl group dilution	0.5
Normal oxygen to be added to give sufficient CO sample	0.15
Overall dilution	$\simeq$ 0.05

As a result of these estimations, it seemed wiser to pyrolyze the total carotenoid fraction as it exists after two chromatographings. The resulting dilution factor of ten means that if the mechanism proposed is operating, the light experiment should yield, after pyrolysis, CO with 0.4% O^{18}, while the dark run should yield CO which is 0.2% O^{18} (normal oxygen value). The actual results are shwon in Table II.

Table II

Sample	Percent O^{18}
1. Original H_2O	4.0%
2. Water in equilibrium with algae: Light run	3.01
3. Water in equilibrium with algae: Dark run	3.06
4. CO sample from xanthophyll fraction of photosynthesized algae (50% carotenoid)	0.245
5. CO sample from xanthophyll fraction of algae kept in dark (40% carotenoid)	0.233
6. Normal H_2O	0.204 (5)

It is seen from this table that both the light and dark samples show an enhancement over normal oxygen. The enhancement can result from O^{18} in the epoxide or furan carotenoids, from the hydroxyl groups present in all xanthophyll pigments, or from the steroid material. It apparently could not result from exchange with

(5) Thode and Smith, National Research Council of Canada, Atomic Energy Project, Report MC-57 (revised), May 1944.

hydroxyl groups already present, however, since such exchanges for ordinary alcohols have been shown to be extremely slow (3). The mass spectrographic analysis was of sufficient accuracy that the additional enhancement of the light run may be regarded as real. Nevertheless, the experiment is inconclusive; it suggests only that it would probably be worthwhile to repeat the work with water of high O^{18} content (30-50%) so that the various carotenoid pigments could be isolated in pure form in sufficient quantities for mass analysis. The use of the high O^{18} content water in such an experiment would make non-existent the difficulties encountered in this experiment, and would permit results to be obtained which might provide a real clue as to the path of oxygen in photosynthesis.

SUMMARY

An experiment is described in which an attempt is made to follow the path of oxygen in photosynthesis by the use of O^{18} as a tracer.